Enhancing Sequence Coverage of Integral Membrane Proteins Using Proteomic Approaches: Application to the G-protein Coupled Receptor for Neurotensin

Jenny TC Ho & Sonja Hess

Mass Spectrometry and Proteomics Facility, NIDDK, NIH, DHHS, Bethesda, MD

Overview

• Apply mass spectrometry based proteomic approaches to help understand the mechanisms involved in the recognition, binding and activation of the G-protein coupled receptor (GPCR) for neurotensin (NTR1).
•To obtain complete sequence coverage of rat NTR1 to monitor conformational changes, characterize mutations in protein sequence and post-translational modifications.

Introduction: GPCRs

•GPCRs are involved in signal recognition and cell communication. Over 1,000 different GPCRs have been identified and many are implicated as therapeutic drug targets.
•Neurotensin is involved in modulatory functions for example in the heart, digestive system and in the central nervous system.
•When the target ligand (for example neurotensin) binds to its GPCR, the receptor undergoes a conformational change, this in turn activates G-proteins and consequently activates or inhibits effector molecules.

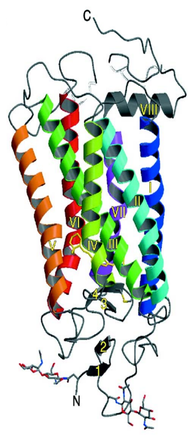

Figure 1: Ribbon drawing of GPCR rhodopsin[2]

•GPCRs are the largest family of receptors. They all have a similar structural framework; seven helices that traverse the lipid membrane linked by intra- and extra-cellular loops[1] (figure 1).
•Analysis of these receptors by MS based proteomic methods are more challenging compared to soluble proteins due to their hydrophobicity.
•These proteins are prone to aggregation and require detergents for solubilization, which can have deleterious effects on LC separation and mass spectral analysis.

References

1.T.P Iismaa *et al.*, G Protein-Coupled Receptors, R.G Landes Company, 1995
2.http://blanco.biomol.uci.edu/Membrane_Proteins_xtal.html
3.J.F White *et al.*, FEBS Letters, 2004, 564, 289-293.

Acknowledgments

Many thanks to R. Grisshammer (NIDDK, NIH) for providing NTR1 and to G. Abdoulaeva, CBER, FDA for synthesizing transmembrane peptide.

Experimental

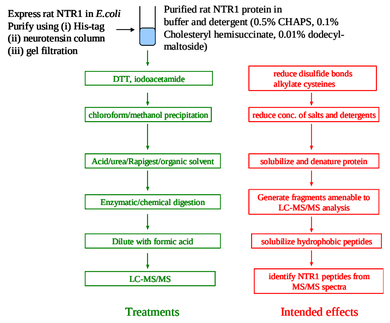

Figure 2: Flow diagram outlining the procedures used to obtain peptide mass map of rat NTR1.

•Rat NTR1 was expressed and purified[3]. Figure 2 shows the procedures used to obtain peptide mass map of NTR1. LC-MS/MS was performed using a NanoAquity LC system (C18 Asymmetry column, ACN containing 0.2% formic acid gradient) and Q-ToF II MS instrument (Water/Micromass).
•Transmembrane peptide mimic (TMpep 1-66) was synthesized. The crude material was solubilized in 50% formic acid and purified on a Zorbax C3 column (Agilent) employing an ACN gradient containing 0.2% formic acid. ES analysis of transmembrane peptides was performed by direct infusion using a Nanomate (Advion) into an LTQ-FTICR (Thermo).

Results I – Optimization of methods using transmembrane peptide mimics

•A bottom-up proteomics approach was employed to obtain complete sequence coverage of NTR1.
•Insufficient sequence coverage of NTR1 was obtained when samples prepared following procedures generally used in proteomic studies. For example, only 15% and 8% of rat NTR1 was observed when digested with trypsin in 8M urea and with proteinase K respectively (figure 3).

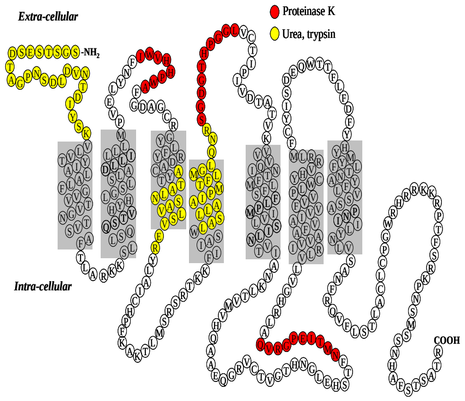

Figure 3: Amino acid sequence of rat NTR1. Yellow and red circles represent residues observed by LC-MS/MS after trypsin digestion in urea and proteinase K digestion, respectively.

• Secondary structure, solubility, peptide length and hydrophobicity may explain why peptides that make up the transmembrane (TM) regions are rarely observed in proteomic analyses. To evaluate these parameters, a peptide that resembles TM regions was synthesized. The AA sequence of the TM peptide mimic (TMpep1-66) and ES spectrum is shown in figure 4. This sequence corresponds to TM I and II of rat NTR1.

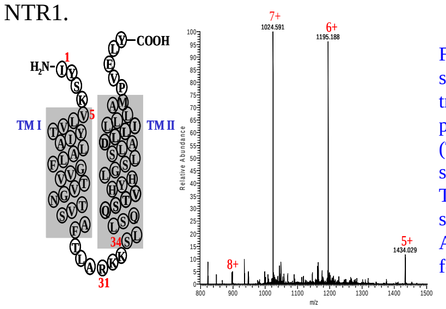

Figure 4: Amino acid sequence of transmembrane peptide mimic (TMpep 1-66). ES spectrum of purified TMpep 1-66, spray solvent 80/20 ACN/H2O, 0.2% formic acid

• Ammonium bicarbonate with the addition of organic solvents eg methanol, acetonitrile or acetone, were investigated to solubilize TMpep 1-66. Organic solvents were chosen that were also compatible with enzymatic digestion, RPLC and MS analysis.
•Figure 5 shows selected ion chromatograms of peptides generated from a trypsin digest of TMpep 1-66 in the presence of 60% methanol. Successful RPLC separation of hydrophobic peptides that make up TM domains was achieved. Peak tailing was more prominent with increasing peptide hydrophobicity.

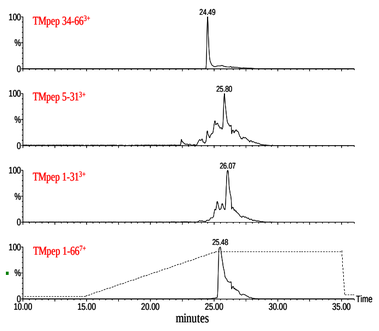

Figure 5: LC-MS analysis of TMpep 1-66 tryptic digest. Selected ion chromatogram of TMpep 1-66^{7+}, TMpep 1-31^{3+} (1 missed cleavage), TMpep 5-31^{3+} and TMpep 34-66^{3+}. Dotted line indicates % of ACN gradient containing 0.2% formic acid.

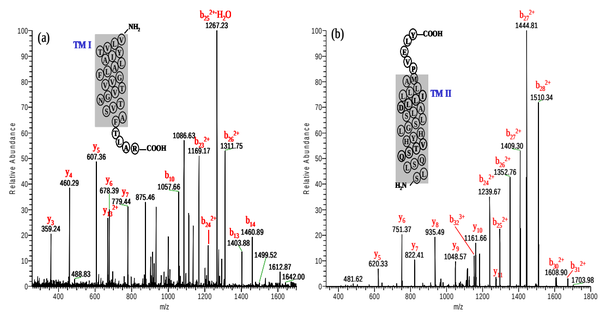

Figure 6: ES MS/MS spectrum of tryptic peptides generated from the digestion of TMpep 1-66 (see figure 4). (a) MS/MS spectrum of TMpep 5-31^{3+} VLVTAIYLALFVVGTVGNSVTAFTLAR (makes up TM I of rat NTR1). (b) MS/MS spectrum TMpep 34-66^{3+} SLQSLQSTVHYHLGSLALSDLLILLLAMPVELY (makes up TM II of rat NTR1).

•Tryptic peptides; TMpep 5-31 and TMpep 34-66 were subjected to CAD in a Q-ToF and LTQ-FT instrument. Figure 6 shows MS/MS spectra of M+3H^{3+} TM peptides acquired using a LTQ-FTICR.

Results II – Mapping NTR1

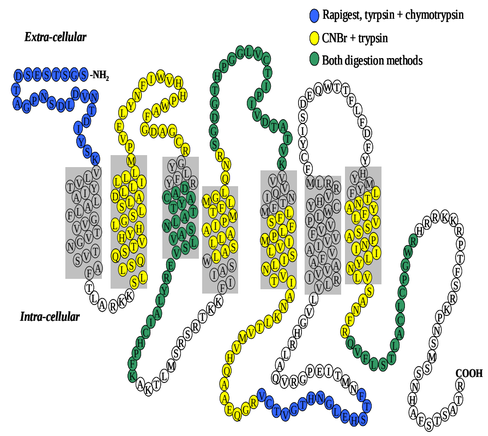

Figure 7: Amino acid sequence of rat NTR1. Blue, yellow and green circles represent residues observed by LC-MS/MS after trypsin+chymotrypsin digestion in Rapigest, CNBr followed by trypsin digestion and in both digestion methods, respectively.

• Methods optimized using a synthetic TM peptide mimic were applied to recombinant rat NTR1. Figure 7 shows the AA sequence of rat NTR1, where the colored circles indicate peptides observed by LC-MS/MS either after digestion with CNBr followed by trypsin or trypsin+chymotrypsin digestion in 5% acid labile surfactant (Rapigest, Waters).
•Peptides corresponding to 5 out of the 7 TM regions have been observed, as well as the intra- and extra-cellular loops.

Conclusions

• The choice of solvent used to prepare integral membrane protein for mass spectral analysis is important. It should (i) solubilize the protein (ii) be compatible with enzymatic/chemical digestion and (iii) be compatible with down stream analysis; LC separation and mass spectral analysis.
• To improve our understanding as to why transmembrane peptides are often under-represented in proteomic analyses, a synthetic peptide was used to optimize sample preparation (i.e test various solubilizing reagents and digestion buffers) and also LC-MS/MS analyses.
•Precipitation of NTR1 reduced concentration of detergents. High % of acid was more effective in solubilizing the precipitated protein compared to organic solvents.
• Using these conditions we are now able to increase sequence coverage from 24% to 60% of the rat NTR1 protein when using multiple enzymes (figure 7).